# CHEMICAL AND BIOLOGICAL SENSING UTILIZING FUSED BACTERIORHODOPSIN PROTEIN HYBRIDS

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## ABSTRACT

This paper describes how monomeric bR can be overproduced in *Escherichia coli* and subsequently utilized as an integral component of a generic, nanoscale chemical sensing platform. The utility of this sensing platform is that it can be adapted for detection of a wide range of biological and chemical agents at, or below, nanomolar concentration levels. The gene encoding for bacteriorhodopsin has been successfully isolated from *Halobacterium salinarum* strain S9P using a colony-level PCR approach. Utilizing this purified DNA and a plasmid expression vector system, a fused protein hybrid consisting of maltose binding protein and bacterio-opsin has been expressed in transformed *E. coli.* The fusion hybrid has been purified in soluble form from *E. coli* cell-free extracts at up to 70mg/L. Renaturation studies to incorporate all-*trans* retinal within the bacterio-opsin protein are currently underway.

## 1. INTRODUCTION

Current chemical sensing systems are cumbersome, not readily deployable, time consuming and generally inadequate for providing the sensitivity required for saving soldiers' lives. The best commercial sensors are currently only able to measure toxin concentrations at the micromolar level, while many biological and chemical agents are lethal at the nanomolar level. This discrepancy shows just how valuable advances in the sensing systems can be to soldiers and civilians of the 21st Century. The evolutionary development of biological systems however, has created an array of natural nanoscale materials with capabilities beyond that of current technology that can be utilized for development of new sensor systems. Bacteriorhodopsin (bR), an optoelectric protein found in the cytoplasmic membrane of *H. salinarum*, is one such material and has been intensely studied over the years due to its intrinsic ability to function as a light-driven proton pump (Haupts *et al.*, 1999). Bacteriorhodopsin strongly absorbs visible light in the 570nm spectral region (Fig. 1) due to a bound prosthetic retinal chromophore that, once excited, undergoes a *trans* to *cis* conformational change. The post-excitation release of energy from the retinal normally facilitates the *in situ* pumping of a proton from the cytoplasmic to the extracytoplasmic side of the cytoplasmic membrane of the organism, thereby creating a voltage potential. This process of excitation, relaxation and proton pumping occurs on the timeframe of 10ms without any refractory period (Hampp, 2000). In *H. salinarum*, the resulting voltage potential is coupled with an ATP synthase to drive the conversion of ADP and $P_i$ to ATP and $H_2O$, thereby providing the energy to drive the

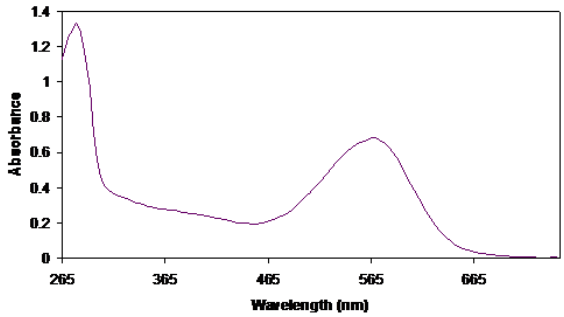


**Fig. 1: UV-VIS Absorbance Spectrum of bacteriorhodopsin.**

cell's internal machinery. Coupling the bR protein with an electrically conductive substrate rather than ATP synthase allows the efficient quantum conversion of light energy into an electric potential. Bacteriorhodopsin is an ideal protein for engineering applications since it is the major component of readily purified purple membrane (PM) (Oesterhelt and Stoeckenius, 1974) and has a partial crystalline structure allowing the protein to remain functional over a wide range of experimental conditions (Hampp, 2000).

For the present study, the production of protein hybrids incorporating bR as a transducing substrate is investigated as well as, the production of monomeric bR. To be utilized as a sensor, the hybrid proteins produced must incorporate a sensing function. This sensing function would require a molecule that is both highly specific for its target, thereby reducing or eliminating false positives, while also binding its target with great tenacity. Although bR lacks an intrinsic sensing mechanism for biological and chemical agents on its own, biological systems are replete with molecules that satisfy these requirements, namely antibodies, enzymes and

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14. ABSTRACT

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binding proteins. These molecules typically bind their targets with high specificity and high affinity (down to attomolar concentrations). The modification of bR in *H. salinarum* is limited, however, the genetic tools for organisms such as *E. coli*, have been developed to such an extent that one may express proteins that are not traditionally found in *E. coli* while also being able to modify the protein at the genetic level prior to transcription, or post-expression. Therefore, for the development of the current proof of concept biosensor, maltose binding protein has been chosen for attachment to the N-terminus of bR by genetic fusion and subsequent expression in *E. coli*.

The maltose binding protein is a protein normally found within the periplasmic space of *E. coli* and binds the disaccharide maltose at attomolar concentrations. Upon binding of maltose, the energy associated with binding induces a 35° conformational change in the MBP, which is the change associated with its transition from an open to a closed state (Jeong, 2006). This energy associated with target molecule binding has been detected in other systems and should also resonate through the MBP-bR fusion protein to perturb the bR established voltage.

To further increase the sensitivity of the device by increasing the perturbation of the bR established voltage potential, fluorescent resonance energy transfer (FRET) between the retinal chromophore, bound within bR, and quantum dots (QD) has been investigated (Griep *et al*, 2007; Je *et al.*, 2005). It has been shown by our group that QDs and bR, within purple membrane patches, can indeed be FRET coupled to allow the non-radiative exchange of energy between the donor, QD, and the acceptor, retinal (Fig. 2). FRET coupling between a

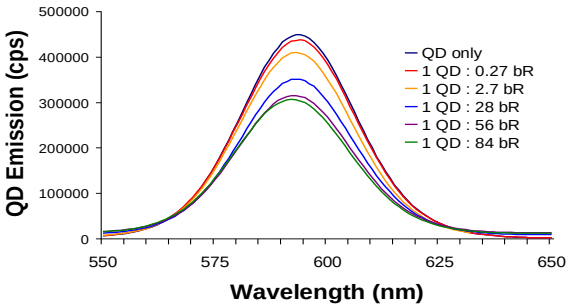


**Fig. 2: Resultant graph showing reduction in QD fluorescent emission upon coupling with bR from PM patches. Proximity achieved with a biotin/streptavidin linking system (Griep, *et al*, 2007).**

donor and acceptor molecule is determined by multiple variables with the two greatest contributing variables the percentage overlap of the donor and acceptor spectra (J-value) and the separation distance between the donor and acceptor (Förster radius), which is a $6^{th}$ power inverse relationship. The production of QDs is such that it allows selection of QDs that provide maximal overlap of the donor and acceptor spectra thereby increasing potential FRET coupling (Fig. 3).

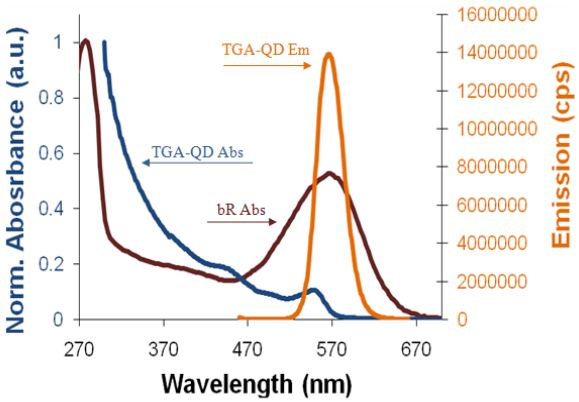


**Fig. 3: Optical absorption and emission properties of size selected TGA-capped CdSe/ZnS QDs along with the absorbance spectra of bR.**

## 2. EXPERIMENTAL

### 2.1 Chemicals

Glucose was purchased from Fisher Scientific (Pittsburgh, PA). Potassium hydroxide, sodium chloride and ampicillin, were obtained from Sigma-Aldrich (St. Louis, MO). Agarose, Lysogeny (Luria) Broth (LB), and Terrific Broth (TB) were supplied by MO BIO (Carlsbad, CA). X-Gal and Taq PCR core kit containing Q-solution was supplied by Qiagen (Valencia, CA). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from EMD Chemicals Inc. (San Diego, CA). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Sequencing buffer and big dye terminator were supplied by Agencourt Technologies (Beverly, MA) and Applied Biosystems (Foster City, CA). High Flow Amylose Resin, *Alw*NI restriction enzyme and Enterokinase were purchased from New England Biolabs (Ipswich, MA). *Bam*HI and *Eco*RI restriction enzymes and T4 DNA Ligase were obtained from Promega (Madison, WI). NuPage® SDS 12% Bis-Tris gels and SimplyBlue™ SafeStain were obtained from Invitrogen (Carlsbad, CA)

**2.2 Media, organisms and growth**

*E. coli* was grown in LB media with constant shaking or on LB plates solidified with 1.5% agar. For selective growth, 200µg/mL ampicillin was added to LB media or 100µg/mL to LB/Agar media. *H. salinarum* strain S9-P was grown according to standard methods (Oesterhelt and Stoeckenius, 1974).

**2.3 Restriction digestions**

Restriction enzyme digestions were conducted according to the manufacturers' protocols. Post-digestion reactions were cleaned by 0.7% agarose gel electrophoresis or by following the solution protocol of the MO BIO GelSpin kit.

**2.4 SDS-PAGE**

SDS-PAGE was performed utilizing 12% NuPage Bis-Tris gels following manufacturer protocols (Invitrogen, Carlsbad, CA) with 10 minute boiling of samples and clarification by centrifugation.

**2.5 Purple membrane purification from *H. salinarum***

Purple Membrane containing bacteriorhodopsin was purified as previously described (Oesterhelt and Stoeckenius, 1974) employing sucrose density gradient centrifugation as the final step. Preparations were evaluated by SDS-PAGE and concentrations determined using an extinction coefficient at OD 570nm ($A_{570}$) equal to 63 $mM^{-1}$ $cm^{-1}$ and a molecular weight of 27kDa. The purified membrane was suspended in ddI water and stored frozen at -80ºC.

**2.6 Electroporation and cell outgrowth**

Electromax™, electrocompetent DH5α cells, were obtained from Invitrogen (Carlsbad, CA). Electroporation was performed utilizing 50µL of electrocompetent cells with 50ng plasmid at 12.5 kV/cm field strength, 25µF capacitor, 200Ω resistor with a time constant equal to 4.8 msec. Phenotypic expression was conducted in super optimal catabolite (SOC) medium for one hour (37°C) before plating on selective media (LB/Ampicillin).

**2.7 DNA sequencing**

DNA sequencing was conducted by the BigDye dideoxy chain terminator sequencing method using pUC/M13 forward and reverse primers obtained from Applied Biosystems (Foster City, CA) for the pGEM-T vector system, or up to 8 different primers for the pEW-bO vector. PCR utilized 99cycles (96°C for 30secs, 43°C for 15secs, and 60°C for 4min). The PCR samples were subsequently cleaned using CleanSEQ according to the manufacturer's protocol (Agencourt, Beverly, MA). Samples were sequenced and analyzed using an ABI Prism 310 Genetic Analyzer along with Applied Biosystems 310 Data Collection software version 3.0. Sequences obtained were analyzed using programs available from the National Center for Biotechnology Information.

**2.8 Bacterio-opsin gene isolation**

The 789-bp gene for bacterio-opsin was isolated from whole cells of *Halobacterium salinarum* utilizing colony-level PCR (Chung-Jui Tsai, Personal Communication). Primers were designed utilizing GeneRunner and IDT OligoAnalyzer 3.0 software and PCR conditions involved 30 cycles ( 94°C for 30secs, 54°C for 45secs, and 72°C for 60secs) followed by a final incubation at 72°C for 5min. PCR products were purified by gel electrophoresis using 0.7% agarose-ethidium bromide gels and products were recovered from the gels using the MO BIO UltraClean™ GelSpin DNA Purification Kit.

**2.9 Induction and purification of fusion protein, MBP-bOmut**

Transformed *E. coli* were grown in selective LB media, with 0.2% Glucose at 37°C with constant shaking, to mid-log phase. At mid-log phase ($OD_{590}$=0.5-0.9) plasmid transcription was induced by addition of IPTG to a final concentration of 0.3mM. Post-induction, cells were kept in the water bath with constant shaking for up to 21 hours and then harvested by centrifugation at 15,000xg for 15min. Subsequent cell lysis and purification followed the protocol designed by (Chen and Gouaux, 1996). During experimentation allowing production of soluble fusion protein, the purification was accomplished by proceeding directly from cell lysis to loading and eluting from an amylose column.

**2.10 Enterokinase digestion**

Enterokinase digestions were performed in 20mM Tris-HCl buffer at pH 8.0 with 0.03% enterokinase to fusion protein (w/w) ratio and placed at RT for 14 days.

## 3. RESULTS AND DISCUSSION

It has been shown that bR patches can be readily isolated from purple membrane and even though bR is the principal component of purple membrane, the uniformity of the membrane is not consistent, therefore, variations in the amount of bR molecules within given samples will undoubtedly occur. To reduce variability in any resultant sensing device, the use of monomeric bR is desired. The production of monomeric bR is possible by expressing the gene for bacterio-opsin in *E. coli* and subsequently

reconstituting the protein into functional bR (Chen and Gouaux, 1996). In this study, the wild-type bacterio-opsin gene from *H. salinarum* strain S9P was isolated for controlled expression in *E. coli.* The PCR primers designed for bacterio-opsin isolation were 18mers and denoted as EW1(F) and EW1(R) (5'-CATGTTGGAGTTATTGCC -3', 5'-GTGTCGTTGAACACAGCC-3') with $T_m$'s equal to 48.5°C and 54.2°C, respectively. These primers incorporated one upstream base and fifty downstream bases flanking the 789-basepair bacterio-opsin gene thereby amplifying the bO gene within an 840-bp amplicon. The resultant PCR products consisted of multiple amplicons with a prominent band, as expected, at 840-bp as shown in Fig. 4.

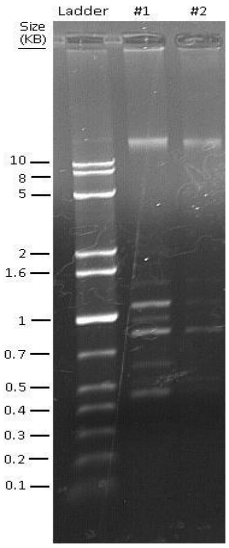


**Fig. 4: Image of 0.7% agarose gel post colony-level PCR of *H. salinarum*. From left to right the lanes contain, molecular weight ladder with sizes (kb) noted to the left and replicate PCR samples (#1 & #2).**

The 840-bp fragment was extracted, purified and subsequently cloned into vector pGEM-T. Sequencing of the 840-bp insert confirmed the presence of the complete 789-bp bO gene within the cloned insert. To allow the in-frame cloning of this insert into the pMal-c4e expression vector, the 5'-terminus of the fragment was modified using the custom designed primer-adaptor sequence, 5'-AATGAATTCATGTTGGAGTTATTGCC-3', which possesses a unique EcoRI restriction site. PCR utilizing this custom forward primer, EW2(F), combined with the reverse primer, EW2(R), produced the DNA fragment designated Eco-bO as shown in Fig. 5. The 3'-terminus of Eco-bO was subsequently subjected to *Alw*NI restriction (New England Biolabs) and the resulting 3bp overhang was annealed and ligated to the dsDNA adaptor EW3(R) (5'-TTTTACTGCAGGATCCTCAGTCG-3') containing BamHI and PstI restriction sites. PCR amplification of this fragment utilizing EW2(R) as the reverse primer and EW2(F) as the forward primer produced DNA fragment EW-bO with an upstream *Eco*RI restriction site and downstream *Bam*HI and *Pst*I restriction sites.

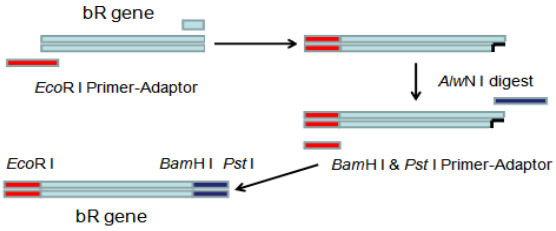


**Fig. 5: Schematic of bacterio-opsin gene manipulations with gene and pertinent restriction sites labeled.**

Double digestion of EW-bO and the pMal-c4e plasmid expression vector with *Eco*RI and *Bam*HI allowed the directional insertion of EW-bO into the expression vector to produce the vector designated pEW-bO. The pEW-bO vector was then introduced into cells of *E. coli* strain DH5α via electroporation and following outgrowth, the transformed cells were plated on selective media and transformants selected by utilization of blue/white screening with X-Gal and IPTG. Transformants were grown overnight in selective TB media for colony-level screening and two transformants were selected for plasmid isolation using a QIAprep Spin MiniPrep kit (Qiagen). This purified plasmid DNA was then subjected to a double digestion using both *Eco*RI and *Bam*HI and analyzed by 0.7% agarose gel electrophoresis. The purified plasmids from both transformants produced a restriction fragment equal to the size of EW-bO upon double-digestion indicating a successful insertion (Fig. 6). The in-frame orientation of the bO gene with MBP was subsequently confirmed by sequencing followed by BLASTn and BLASTx analysis.

In anticipation of expression issues, alternate frame translation initiation start sites present in the bO gene were investigated and site-directed mutagenesis was utilized to remove these alternate start sites by introducing silent point mutations. To obtain these mutations, new custom PCR primers were designed to produce amplicons with single base changes at bO bases: 152, 447 and 720. This amplicon was extended in size by utilizing itself as either the forward or reverse primer in a new round of PCR complemented by another primer outside the amplicon region and continuing outward until all sites were mutated and the full construct amplified. The resultant fragment was denoted as EW-bOmut and directionally inserted into the pMal-c4e expression vector, as was EW-bO, and subsequently inserted into *E. coli* strain DH5α via electroporation. These putative transformants were then screened using X-Gal and IPTG and by colony-level PCR to obtain positive transformants.

Screened transformants were shown to possess plasmids with inserts and the identities of the inserts were confirmed by sequencing.

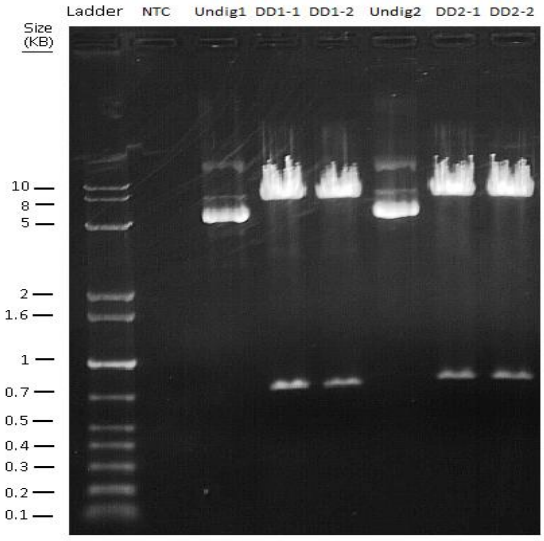


**Fig. 6: 0.7% agarose gel image of pEW-bO transformants and double digestion samples. From left to right wells contain: 1Kb Plus Ladder, non-template control (NTC), undigested purified plasmid 1, replicate double-digests of plasmid 1, undigested purified plasmid 2, and replicate double-digests of plasmid 2.**

Following confirmation of the inserted plasmid sequences, multiple induction studies were performed on the *E. coli* transformants with induction times varying from 3 hours to 21 hours. Two distinctly different results have been obtained from these studies. First, during short inductions (3 hours), there is little, if any, of the fusion protein present in the soluble cell lysis fraction, which is in agreement with previous findings (Chen and Gouaux, 1996).Utilizing an inclusion body solubilization procedure (Chen and Gouaux, 1996) the fused protein was recovered at 10mg/L of cells. Secondly, with induction times of 17 hours and longer the fused protein construct occurs in the soluble cell lysis fraction rather than in the insoluble fraction. With the fused protein construct present in soluble form the protein can be directly purified by use of an amylose affinity chromatography column (Fig. 7A) with recoveries up to 70mg/L of cells.

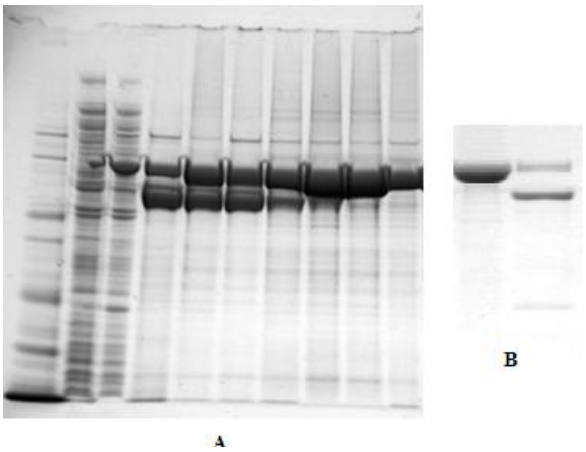


**Fig. 7: A) SDS-PAGE image of not induced and 17-hr induced whole cell preps from transformed cells, lanes 2 and 3, respectively with lanes 4-10 amylose column elution samples from the soluble cell lysis fraction. B) SDS-PAGE image of protein sample before (lane 1) and after (lane 2) enterokinase digestion.**

As shown in Fig. 7A, the fusion protein migrates at 55 kDa, which is lower than the expected size of 61-67 kDa, which is the combined size of MBP (40kDa) and bR (27kDa but migrates on SDS at 21kDa). This 55kDa band however is lost upon digestion of the sample with enterokinase, which selectively cleaves at the fusion between MBP and bR. The loss of the fusion 55kDa protein can be seen in Fig. 7B showing the control protein sample and the sample post-digestion where the 40kDa band (identical to MBP) and a 21kDa band (identical to bR) appear.

## CONCLUSIONS

The unique photoelectrical properties, ease of preparation, and robust nature of bacteriorhodopsin render this protein ideal for applications at the nanoscale level. The MBP-bR system will serve as a proof-of-concept system for the utility of using a fused sensor-bR hybrid protein to modulate biosensor electrical output upon target molecule binding. It is proposed that such a hybrid protein may be directly substituted for bR as the transducing substrate in a biosensing platform. The broader impact of this system is that the biosensor can be easily altered to specifically target different chemical agents by interchanging the maltose binding protein portion of the hybrid with a different binding protein, enzyme or antibody. The production of this protein using vector pEW-bOmut, constructed in the present study, which at present can yield up to 70mg/L of the fused MBP-bO protein represents a big step forward in the production of fused sensor proteins or monomeric bR.

The current yield of bR from *H. salinarum* is approximately 4mg/L of cells and requires one week of growth prior to harvest.

High-level production of the apoprotein bO, in *E. coli* is achieved overnight however, the retinal that is naturally produced in *H. salinarum* is not produced in *E. coli* and must be introduced after protein purification. Renaturation studies of the fused MBP-bO protein produced are currently underway to incorporate all-*trans* retinal and subsequently yield MBP-bR protein. Once renatured, further tests will be conducted to confirm photoelectrical activity and test the influence associated with binding of the target molecule, maltose, on an established electrical signal.

The energy associated with target molecule binding has been detected in other systems and should also resonate through the MBP-bR fusion protein to perturb the bR established voltage. This perturbation of the signal would be indicative of a positive binding event and provide confirmation of the chemical in the environment in less than 10ms. A response time of ten milliseconds would represent a dramatic decrease in the minutes to hours to days required for current chemical sensing systems to confirm the presence of a target molecule.

Due to the genetic tools available, the bR protein can be quickly modified at the DNA level to allow the production of fusion proteins with virtually any sensor protein required for targeting of a specific chemical, or biological agent, be that Agent Orange, anthrax, cyanide, or other agent. Additionally, by deposition of the fused bR hybrid protein onto an individually addressable electronic device, sensing array platforms could be custom developed for specific applications permitting an even greater increase in civilian and soldier security.

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